Immunogene Therapy of Tumors with Vaccine Based on Xenogeneic Epidermal Growth Factor Receptor

You Lu, Yu-quan Wei, Ling Tian, Xia Zhao, Li Yang, Bin Hu, Bin Kan, Yan-jun Wen, Feng Liu, Hong-xin Deng, Jiong Li, Yong-qi Mao, Song Lei, Mei-juan Huang, Feng Peng, Yu Jiang, Hao Zhou, Li-qun Zhou and Feng Luo

J Immunol 2003; 170:3162-3170; 
doi: 10.4049/jimmunol.170.6.3162
http://www.jimmunol.org/content/170/6/3162

Why The JI?
• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication

References
This article cites 58 articles, 36 of which you can access for free at:
http://www.jimmunol.org/content/170/6/3162.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2003 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Immunogene Therapy of Tumors with Vaccine Based on Xenogeneic Epidermal Growth Factor Receptor

You Lu,* Yu-quan Wei, Ling Tian,* Xia Zhao,† Li Yang,* Bin Hu,* Bin Kan,* Yan-jun Wen,* Feng Liu,* Hong-xin Deng,* Jiong Li,* Yong-qi Mao,* Song Lei,* Mei-juan Huang,* Feng Peng,* Yu Jiang,* Hao Zhou,* Li-quin Zhou,* and Feng Luo*

The breaking of immune tolerance against self epidermal growth factor receptor (EGFr) should be a useful approach for the treatment of receptor-positive tumors with active immunization. To test this concept, we constructed a plasmid DNA encoding extracellular domain of xenogeneic (human) EGFr (hEe-p) or corresponding control mouse EGFr (mEe-p) and empty vector (c-p). Mice immunized with hEe-p showed both protective and therapeutic antitumor activity against EGFr-positive tumor. Sera isolated from the hEe-p-immunized mice exhibited positive staining for EGFr-positive tumor cells in flow cytometric analysis and recognized a single 170-kDa band in Western blot analysis. Ig subclasses responded to rEGFr proteins were elevated in IgG1, Ig2a, and Ig2b. There was the deposition of IgG on the tumor cells. Adoptive transfer of the purified Igs showed the antitumor activity. The increased killing activity of CTL against EGFr-positive tumor cells could be blocked by anti-CD8 or anti-MHC class I mAb. In vivo depletion of CD4+ T lymphocytes could completely abrogate the antitumor activity, whereas the depletion of CD8+ cells showed partial abrogation. The adoptive transfer of CD4-depleted (CD8+) or CD8-depleted (CD4+) T lymphocytes isolated from mice immunized with hEe-p vaccine showed the antitumor activity. In addition, the increase in level of both IFN-γ and IL-4 was found. Taken together, these findings may provide a new vaccine strategy for the treatment of EGFr-positive tumors through the induction of the autoimmune response against EGFr in a cross-reaction between the xenogeneic homologous and self EGFr. The Journal of Immunology, 2003, 170: 3162–3170.

Epidermal growth factor receptor (EGFr) gene encodes a Src family receptor tyrosine kinase with oncogenic potential (1, 2) and often triggers a cascade of cellular biochemical events leading to cell growth and differentiation in the presence of the binding-ligands (3). Increased numbers of EGFr can markedly influence the growth properties of cells and lead to develop tumor in nude mice in an EGFr-dependent manner (4, 5). In addition, autocrine and paracrine growth factor stimulation of the tumor is predominantly mediated by an EGFr-related pathway (6), which can result in the growth advantage of tumor cells. Accumulating evidence shows that EGFr is implicated in the progression of tumor. Moreover, the overexpression of EGFr has been detected in many human tumors, including non-small cell lung cancer (NSCLC) (7–9), cancer of the breast (10), the bladder (11, 12), and the brain (13). High levels of expression of this receptor also have been associated with spread of NSCLC (8), invasion and metastasis of bladder cancer (11, 12), and poor survival in some patients of breast cancer (10). Clearly, evaluation of EGFr as therapeutic targets for tumor is highly warranted. The extracellular domain of EGFr was often targeted by administration of mAbs that are capable of inhibiting tumor growth through some direct or indirect mechanisms in previous studies (14, 15). Passive immunization with anti-EGFr mAb is undergoing in clinical trials (14, 15).

The breaking of immune tolerance against self EGFr and induction of autoimmunity against receptor-positive tumors should be another useful approach for the treatment of EGFr-positive tumor cells with active immunization. However, the immunity to self EGFr is presumably difficult to elicit by vaccines based on self or syngeneic EGFr due to the immune tolerance acquired during the development of the immune system. Sequence comparison analysis by searching Swissprot database in the National Center for Biotechnology Information in the present study indicates that the extracellular domain of human EGFr and that of mouse EGFr molecule are 88% identical at amino acid level, indicating that this molecule is highly conserved during evolutionary process. The current studies explore the feasibility of immunogene therapy of EGFr-positive tumors with the plasmid DNA encoding xenogeneic EGFr as vaccine by the breaking of the immune tolerance against EGFr in a cross-reaction between the xenogeneic homologous and self EGFr.

To test this concept, we constructed a plasmid DNA encoding extracellular domain of human EGFr (hEe-p). At the same time, the plasmid DNA encoding the corresponding extracellular domain of mouse EGFr (mEe-p) and empty vector (c-p) were also constructed and used as control groups. The DNA vaccines were tested for the ability to induce antitumor immunity in EGFr-positive lung cancer and breast cancer in a mouse model. In the
present study, the plasmid DNA encoding xenogeneic EGFr as vaccine is shown to be effective in both protective and therapeutic antitumor immunity, in which autoreactive immunity may be directed against the EGFr-positive tumor cells in both humoral and cellular immunity. These observations may provide a new vaccine strategy for active immunotherapy by the breaking of immune tolerance against EGFr in a cross-reactivation.

Materials and Methods

Cells lines

A549 human lung carcinoma, K562 human chronic myeloid leukemia, and YAC-1 were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and grew in RPMI medium 1640 with 10% FBS. LL/2c murine Lewis lung carcinoma and MMT-06052 murine mammary carcinoma were also purchased from ATCC and grew in DMEM supplement with 10% FBS. These tumor cell cultures were maintained in a 37°C incubator with a humidified 5% CO₂ atmosphere.

Plasmid DNA vaccine constructs and preparation

Cultured cells, including A549 and LL/2c, were harvested and total RNA was isolated using a high pure RNA isolation kit (Boehringer Mannheim, Indianapolis, IN), respectively. Each of the RNA was subjected to RT-PCR for amplification of the extracellular domain of EGFr using one-step RT-PCR kit (Boehringer Mannheim). Flanking primers for an extracellular domain of human EGFr (hEe) were GCTATGGAGGAAAAGAAAGTTTGCAAA and ATGGAGGTTTCATGGGCTCTTGCT. The primers for that of mouse EGFr (mEe) were GCCATGGAGGAAGAAGAAGTAATGGTGGCAAA and AATAGATGTTATTTTCTTTGCGCAGAG. The amplified products were inserted into pT-Adv (Gene Company, Hong Kong, China) and then subcloned into pcDNA3.1 (Invitrogen, San Diego, CA), which contains a CMV promoter. The extracellular domains of human and mouse were inserted into pcDNA3.1 to construct the expression vectors, hEe-p and mEe-p, respectively. As a control, pure pcDNA3.1 plasmid was used as empty vector (c-p). The sequence of extracellular domains of human and mouse EGFr was confirmed by dideoxy sequence to be identical with those previously reported (1, 4). The expression of plasmid DNA was confirmed in the transfected cells by using RT-PCR and by anti-EGFr Abs in Western blot analysis and ELISA. Plasmids for DNA vaccination were purified by using two rounds of passage over Endo-free columns (Qiagen, Chatsworth, CA), as previously reported (16, 17).

Tumor models

LL/2c Lewis lung cancer and B16 melanoma were established in C57BL/6. MA782/5S mammary cancer, Meth A fibrosarcoma, and H22 hepatoma models were established in BALB/c mice (17, 18). All studies involving mice were approved by the Institute’s Animal Care and Use Committee.

DNA immunization

Mice were immunized with different doses (5–150 μg/mouse) of hEe-p, mEe-p, or c-p in normal saline by i.m. injection (17), respectively. Additional control animals were injected with normal saline. Mice were bled via the tail vein at weekly intervals to assess titer of anti-EGFr Abs.

Immunohistochemistry and flow cytometric analysis

Immunohistochemistry was described (19). Frozen sections were fixed in acetone, washed with PBS, and incubated with goat FITC-conjugated Ab against mouse IgA, IgM, or IgG (Sigma-Aldrich, St. Louis, MO). Slides were examined by fluorescence microscopy. For the observation of potential side-effects in the treated mice, the tissues of heart, liver, spleen, lung, kidney, brain, etc. were also fixed in 10% neutral-buffered formalin solution and embedded in paraffin. Sections 3–5 μm were stained with H&E.

In flow cytometric analysis, tumor cells were stained by an indirect method (18, 20) using 1:50 to 3000 diluted serum, and then FITC-goat anti-mouse IgG, IgM, and IgA (Sigma-Aldrich). In addition, rabbit polyclonal anti-EGFr (mouse, rat, and human reactive) (Santa Cruz Biotechnology Santa Cruz, CA) was used as the control Ab to identify the expression of EGFr. In addition, EGFr-positive tumor cells were also confirmed in Western blot analysis with the commercially available Ab.

Western blot analysis

Western blot analysis was performed as described (18, 20). Briefly, 2 × 10⁶ cells were lysed in 1 ml lysis buffer. The samples were denatured in sample buffer and boiled at 100°C for 5 min. Proteins were separated by SDS-PAGE. Gels were electroblotted with Sartoblot onto a polyvinylidene difluoride membrane. The membrane blots were blocked in 4% nonfat dry milk, washed, and probed with mouse sera at 1:200. Blots were then washed and incubated with a biotinylated secondary Ab (biotinylated

FIGURE 1. Induction of the protective antitumor immunity. Mice (15 mice in each group) were immunized i.m. with 100 μg of hEe-p, mEe-p, c-p, or nonimmunized (saline alone) once a week for 4 wk. Mice were then challenged with 5 × 10⁵ LL/2c Lewis lung cancer (A and C) or MA782/5S mammary cancer cells (B and D) s.c. 1 wk after the fourth immunization. A and B. Tumor volume (mm³) in mice treated with hEe-p and controls was shown. C and D. Percentage survival of mice treated. The hEe-p-treated mice have also been followed for >5 mo. The survival rate of the mice was 60–66% at day 150 for LL/2c Lewis lung cancer and MA 782/5S mammary cancer, respectively.

FIGURE 2. Induction of the therapeutic antitumor immunity. Mice (15 mice in each group) treated with i.m. injection of 100 μg of hEe-p, mEe-p, c-p or saline alone once weekly for 4 wk starting at day 5 after 1 × 10⁶ live LL/2c (A) and MA782/5S (B) cells were introduced s.c. into mice. The hEe-p-treated mice have also been followed for >5 mo. The survival rate of the mice was 40%, and 53% at day 150 for LL/2c Lewis lung cancer and MA 782/5S mammary cancer, respectively.

Downloaded from http://www.jimmunol.org/ by guest on November 13, 2017
The viability of cells was determined by a trypan blue dye exclusion test, and the previously detailed (21).

Experimental mouse sera were serially diluted and added to wells. Plates were incubated for 2 h at 37°C overnight. Nonspecific binding was blocked with 2% milk powder in PBS plus Tween 20 for 2 h. The recombinant extracellular domain protein of human or mouse EGF was expressed in insect cells and purified from the cell supernatant by sequential metal chelate affinity chromatography and gel filtration, as previously detailed (21).

**Ig subclass response to rEGF protein**

The Ig subclass was determined by ELISA as described (17). Briefly, microtiter plates were coated with the recombinant extracellular domain protein of human or mouse EGF (0.5 μg/well) in 100 μl coating buffer (sodium carbonate, pH 9.6) and incubated at 4°C overnight. Nonspecific binding was blocked with 2% milk powder in PBS plus Tween 20 for 2 h. Experimental mouse sera were serially diluted and added to wells. Plates were incubated for 2 h at 37°C, washed, and then incubated with serially diluted alkaline phosphatase-conjugated anti-mouse IgG subclass or anti-IgM or anti-IgA. Enzyme activity was measured with an ELISA reader (Bio-Rad, Richmond, CA).

The recombinant extracellular domain protein of human or mouse EGF was expressed in insect cells and purified from the cell supernatant by sequential metal chelate affinity chromatography and gel filtration, as previously detailed (21).

**Purification of Ig, its inhibition of cell proliferation in vitro, and its adoptive transfer in vivo**

Igs were purified from the pooled sera derived from the mice on day 7 after the fourth immunization or from control mice by affinity chromatography (CM Affi-Gel blue gel kit, Bio-Rad). For the determination of the effects of purified Igs on cell proliferation, exponentially growing EGF-p-positive or -negative tumor cells, at a concentration of 2 × 10^5/mL, were exposed to various concentrations (1–1000 μg/ml) Igs for 72 h culture. The number of viable cells was determined by a trypan blue dye exclusion test, and the percentage inhibition was calculated (18).

To assess the efficacy of Ig in antitumor in vivo, the purified Ig (10–300 mg/kg) was adoptively i.v. transferred 1 day before mice were challenged with 1 × 10^3–1 × 10^5 tumor cells and then treated twice per week for 3 wk. As a control, Ig was adsorbed four times by the incubation with the fixed EGF-p-positive or -negative tumor cells for 1 h at 4°C with rocking (18).

**FIGURE 4.** Identification of autoantibodies against EGF-p-positive tumor cells. A–E. Staining for the tumor cells in flow cytometric analysis. LL/2e (A), MA782/5S (B), A549 (C), and K562 (D) cells were stained by an indirect method using 1:500 diluted sera and then FITC-goat anti-mouse IgG in flow cytometric analysis. LL/2e (A), MA782/5S (B), and A549 (C) showed positive staining for sera isolated from hEe-p-immunized mice. In contrast, K562 cells (D) showed negative staining. In addition, these cells showed negative staining for the sera isolated from the nonimmunized mice and other control groups. A representative histogram (E) from LL/2e cells showed negative staining for the sera isolated from the nonimmunized mice.

**Cytokine response**

IL-4 and IFN-γ were determined as previously described (22, 23). Briefly, splenocytes obtained from the immunized or control mice were treated with ammonium chloride-potassium lysing buffer to deplete erythrocytes. To prepare T-enriched cell fraction, these splenocytes were further incubated in complete medium for 90 min, and nonadherent cells were removed gently and incubated on nylon wool columns. Recovered T-enriched cells were washed and plated at 2 × 10^5 cells per well in flat-bottom microtiter plates. These T cells were restimulated with tumor cell lysate-pulsed APCs (2 × 10^5/well) as previously described (23). After 48 h at 37°C, 100 μl of supernatant was harvested and tested for IFN-γ and IL-4 by ELISA kit (BD PharMingen, San Diego, CA).

**In vitro cytotoxicity assay**

A 4-h 51Cr release assay was performed as described and previously described by other reports (20, 23). Briefly, splenocytes obtained from the immunized or control mice were treated with ammonium chloride-potassium lysing buffer to deplete erythrocytes. T-enriched cell fraction was prepared as previously described. A total of 100 μl of effector cells and 31Cr-labeled target cells were assigned at different E:T ratios to each well of microtiter plates and incubated for 4 h at 37°C. Samples were then harvested, and the activity was calculated by the formula: % cytotoxicity = [(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 100. To evaluate NK activity, splenocytes treated with ammonium chloride-potassium lysing buffer to deplete erythrocytes were used as effectors against YAC-1 NK target cells. The spontaneous 31Cr release usually did not exceed 20% of the total isotope count.

In the cytotoxicity inhibition assays, effectors cells or 31Cr-labeled tumor cells were pretreated with mAb at room temperature for 30 min, washed, and tested (20, 23). mAbs used included anti-CD4 (10 μg/ml), anti-CD8 (10 μg/ml), anti-H-2Kb/H-2Dd (50 μg/ml) (BD PharMingen). The above concentrations of mAb were effective in mediating their activity in preliminary experiments. Control cytotoxicity assay was performed in the presence of control mAb (anti-H-2Dd) or isotype IgG.

**FIGURE 5.** Western blot analysis for the Ags recognized by the immune sera. A. A549, K562, LL/2c, MA782/5S, MMT (MMT-06052), and H22 cells were stained with sera isolated from mice immunized with hEe-p. A single 170-kDa band can be recognized with the sera in EGFp-positive tumor cells (A549, LL/2c, and MA782/5S), but not in EGF-p-negative tumor cells (K562, H22, and MMT-06052 cells). B. The cells shown in A stained with sera from nonimmunized mice showed negative staining.
FIGURE 6. Identification of autoantibodies on the tumor cells by fluorescence microscopy. There was the deposition of autoantibodies on LL/2c tumor cells (A) and MA782/5S tumor cells (B) from hEe-p-immunized mice, but not on the corresponding tumor cells from nonimmunized mice (C and D). The mice depleted of CD4⁺ T lymphocytes were immunized with hEe-p and did not develop detectable IgG-specific fluorescence on LL/2c tumor cells (E) and MA782/5S tumor cells the tumor cells (F). In contrast, the depletion of CD8⁺ lymphocytes (G and H) or NK cells showed no effect. Depletion of immune cell subsets was described in Materials and Methods. There was no deposition of autoantibodies within the tissues of the liver (I) and kidney (J) of mice immunized with hEe-p and the corresponding tissues (K and L) from nonimmunized mice.

**Purification of CD4⁺ or CD8⁺ T cells in vitro and their adoptive transfer in vivo**

Purified CD4⁺ or CD8⁺ T lymphocytes from nylon wool-purified splenic T cells were obtained by the treatment with specific Abs plus complement, as previously reported (24, 25). Briefly, nylon wool-purified splenic T cells (10⁵ cells/ml) in complete medium were incubated on ice for 45 min with previously determined optimal amounts of purified mAb to mouse CD4 (10 µg/ml, clone GK 1.5; ATCC) or anti-CD8 (10 µg/ml, clone TIB 211; ATCC). The cells were then washed with RPMI 1640 medium supplemented with 5% FBS. The lymphocytes were then incubated at 37°C for 1 h in the presence of complement (rabbit Low-Tox-M; Cedarslane Laboratories, Hornby, Ontario, Canada). Viable cells were recovered by centrifugation through a 1.119 g/cm³ Ficoll gradient (http://www.jci.org/cgi/ inline-sigma; Sigma-Aldrich), and were washed twice in complete medium before counting (>95% viable by trypan blue exclusion). γδ T cells and NK cells were also depleted using anti-TCR-γδ (clone UC7-13D5; BD PharMingen) and anti-NK1.1 (clone DX5; BD PharMingen) plus complement as previously described. Furthermore, Abs plus complement-treated cells were removed of B cells and adherent cells by passing on anti-mouse IgG-coated dishes, as previously described (25). Depletion of immune cell subsets was confirmed by flow cytometry (Coulter Elite ESP; Coulter, Miami, FL) using FITC-labeled anti-CD4, anti-CD8, anti-NK, and anti-γδ T (BD PharMingen) (20). In all cases, the specific cellular depletions were ≥99% effective. One day after the adoptive transfer of 2 × 10⁶-2 × 10⁷ cells, the mice were challenged with 1 × 10⁵-10⁶ tumor cells.

**In vivo depletion of immune cell subsets**

Immune cell subsets were depleted as described (18, 26). Briefly, mice (10 mice/group) were vaccinated four times weekly with 100 µg of hEe-p, mEe-p, and c-p, or nonimmunized and challenged with 1 × 10⁶-10⁷ tumor cells on day 7 after the fourth immunization. Mice were injected i.p. with 500 µg of either the anti-CD4 (clone GK 1.5, rat IgG), anti-CD8 (clone 2.43, rat IgG), anti-NK (clone PK136) mAb, or isotype controls 1 day before the first immunization, and then twice per week for 3 wk. These hybridomas were obtained from the ATCC. Peripheral blood was collected from two representative mice from each group, and lymphocytes were analyzed by flow cytometry (Coulter Elite ESP) (20) for the depletion of the appropriate cell subset. As a result, the depletion of CD4⁺, CD8⁺, and NK cells was consistently >98%.

**Statistical analyses**

For comparison of individual time points, ANOVA and an unpaired Student t test were used (27). Survival curves were constructed according to the Kaplan-Meier method (28). Statistical significance was determined by the log-rank test (29).

**Results**

*Induction of the antitumor immunity*

To investigate the protective antitumor immunity, we immunized mice by the i.m. injection of plasmid (hEe-p, mEe-p, or c-p) or saline alone (nonimmunized mice) once a week for 4 wk, and then challenged mice with tumor cells. As shown in Fig. 1, tumors grew progressively in mEe-p- or c-p-immunized mice and in all nonimmunized mice (saline alone), but there was apparent protection from tumor growth in hEe-p-immunized mice. The survival of the mice injected with hEe-p was also significantly greater than that of untreated mice or mEe-p- or c-p-immunized mice (p < 0.005, by log-rank test). The protective effect was dose dependent. The dose (100 µg/mouse) used in Fig. 1 is an optimal one selected for the immunization in several preliminary experiments. Treatment with a 150-µg dose did not show greater effect than that with 100 µg. Treatment with a 5- to 15-µg dose shows little effect. In addition, the protective effect with the immunization of hEe-p was not found in EGFr-negative tumors such as H22 hepatoma and MM7-0652 murine mammary carcinoma cells.

The therapeutic efficacy of the DNA vaccine encoding xenogeneic EGFr was next tested in the established tumors. The mice were treated starting at day 5 after the injection of LL/2 lung cancer cells or MA782/5S mammary cancer cells, when the tumor was palpable. The survival of the tumor-bearing mice treated with hEe-p was also significantly greater than that of the controls (p < 0.005, by log-rank test) (Fig. 2).

The mice immunized with these vaccines have been in particular investigated for the potential long-term toxicity. No adverse consequences were indicated in gross measures such as weight loss (Fig. 3A), ruffling of fur, life-span (Fig. 3B). Furthermore, no pathologic changes of liver, kidney, lung, spleens, brain, etc. were found by the microscopic examination. Also, there was no deposition of autoantibodies in major organs examined by immunofluorescence staining (see Fig. 6).
Characterization of autoantibodies against EGFr and their antitumor efficacy

In an attempt to explore the possible mechanism by which the antitumor activity was induced with hEE-p, we stained EGFr-positive and EGFr-negative tumor cells with sera isolated from hEE-p-immunized mice and control mice in flow cytometric analysis. As a result, EGFr-positive tumor cells showed the positive staining with sera from hEE-p (Fig. 4, A–E), but negative staining for sera from mEE-p- or c-p-immunized or unimmunized mice. The positive staining can be found by using FITC-goat anti-mouse IgG as secondary Ab, but not by anti-mouse IgM or IgA. In addition, EGFr-negative tumor cells did not show positive staining for sera from hEE-p or other controls.

Sera from immunized mice were assayed for the presence of EGFr-specific autoantibodies in Western blot analysis. Only sera from mice immunized with hEE-p recognized a single 170-kDa band in EGFr-positive tumor cells, but not in EGFr-negative tumor cells (Fig. 5, A and B).

To identify the possible deposition of autoantibodies within tumor tissues or normal tissues, we investigated the tissues by immunofluorescence staining. As shown in Fig. 6, A–D, there was the deposition of IgG on the tumor cells from hEE-p-immunized mice, but not on the tumor cells from the immununized or mEE-p- or c-p-immunized mice. Furthermore, the mice depleted of CD4+ T lymphocytes were immunized with hEE-p and did not develop detectable IgG-specific fluorescence on the tumor cells (Fig. 6, E–H).

In contrast, the depletion of CD8+ lymphocytes or NK cells showed no effect. Also, no IgM- or IgA-specific fluorescence was found. In addition, no detectable deposition of autoantibodies was found within the major organs such as liver, kidney, spleen, brain, etc. in the immunized or control mice (Fig. 6, I–L).

Ig subclass response to the recombinant extracellular domain protein of mouse or human EGFr was also determined by ELISA and found to be elevated significantly in IgG1, IgG2a, and IgG2b with little increase in IgM or IgA level in sera obtained from the mice at day 7 after the fourth immunization, compared with the controls (Fig. 7). To explore the role of immune cell subsets in Ig subclass response, we treated mice by i.p. injection of either the anti-CD4, anti-CD8, anti-NK mAb or isotype controls at 1 day before the first immunization, and then twice per week for 3 wk. Sera were also collected from the mice at day 7 after the fourth immunization. The mice depleted of CD4+ T lymphocytes did not develop detectable Abs against the recombinant extracellular domain protein of mouse or human EGFr (Fig. 7). In contrast, the depletion of CD8+ lymphocytes or NK cells showed no effect (Fig. 7). These data suggest that the induction of the Ab against EGFr may be involved in CD4+ T lymphocytes.

Furthermore, the treatment with Igs from mice immunized with hEE-p resulted in apparent inhibition of proliferation of EGFr-positive tumor cells, compared with those from mice immunized with mEE-p and c-p or nonimmunized mice (Fig. 8A and B). In contrast, the treatment had no effect on proliferation of EGFr-negative tumor cells (Fig. 8A). Furthermore, adoptive transfer of the purified Igs isolated from hEE-p-immunized mice was effective in protection from the tumor growth (Fig. 8C). Adsorption of Igs with EGFr-negative tumor cells before adoptive transferring could abrogate its antitumor activity, but EGFr-negative tumor cells had no effect, indicating that EGFr-specific Abs may be involved in the antitumor activity (Fig. 8C).
FIGURE 9. Representative experiment of CTL-mediated cytotoxicity in vitro and adoptive transfer of T cell subsets. A, T cells derived from the spleens of hEe-p-immunized mice were tested against LL/2c cells at different E:T ratios by a standard 4-h 51Cr release assay as described in Materials and Methods. T cells derived from the spleens of hEe-p-immunized mice showed higher cytotoxicity against LL/2c cells than did T cells from mEe-p, c-p, or nonimmunized mice by a standard 4-h 51Cr release assay. hEe-p-induced tumor killing activity can be blocked by anti-CD8 or anti-MHC class I (anti-H-2Kb/H-2Db) mAb. B and C, T cells were isolated from spleens of C57BL/6 mice, immunized with hEe-p, m-Ee-p, and nonimmunized mice, and were depleted of CD4+ or CD8+ lymphocytes, as described in Materials and Methods. The adoptive transfer of 2 \times 10^7 CD4-depleted (CD8+) (B) or CD8-depleted (CD4+) (C) T cells from mice immunized with hEe-p showed the antitumor activity against EGFr-positive LL/2c. D and E, The adoptive transfer of 2 \times 10^7 CD4-depleted (CD8+) (D) or CD8-depleted (CD4+) (E) T cells from BALB/c mice immunized with hEe-p was also found to be effective in EGFr-positive MA782/5S mammary cancer model. There was no antitumor activity found in syngeneic EGFr-negative tumor (B16 and Meth A) (B–E). In addition, the transfer of T lymphocyte subsets from mice immunized with mEe-p and nonimmunized mice had no effect (B–E). Data represent day 25 after tumor cell injection. Similar results can be found at other time points.

Cellular immune response in hEe-p-induced antitumor activity

We found that there was no antitumor activity induced by hEe-p in the nude mice, suggesting that T cells may be required for the antitumor response. Next, the tumor killing activity of CTLs were examined. T cells isolated from spleens of hEe-p-immunized mice exhibited higher cytotoxicity against EGFr-positive syngeneic tumor cells than those from control groups (Fig. 9A). This cytotoxicity could be blocked by anti-CD8 or anti-MHC class I mAb, but not by anti-CD4 in vitro (Fig. 9A), suggesting that the killing activity observed may result from MHC class I-dependent CD8+ CTL activity. In addition, there is no increase in NK activity against YAC-1 target cells by the sensitized spleen cells. Furthermore, the adoptive transfer of CD4-depleted (CD8+), or CD8-depleted (CD4+) T lymphocytes isolated from mice immunized with hEe-p vaccine showed the antitumor activity against EGFr-positive tumor cells, but not against the other syngeneic EGFr-negative tumor (Fig. 9 B–E). The transfer of T lymphocyte subsets from mice immunized with mEe-p or nonimmunized mice had no effect (Fig. 9 B–E).

Furthermore, in vivo depletion of CD4+ T lymphocytes could completely abrogate the antitumor activity with the immunization of hEe-p (Fig. 10A), whereas the depletion of CD8+ lymphocytes showed partial abrogation of the antitumor activity in vivo. In addition, the treatment with anti-NK mAb or normal rat IgG showed no effect. Mice depleted of CD4+ T lymphocytes did not develop detectable CTL activity (Fig. 10B) and autoantibodies (Figs. 6 and 7), whereas mice depleted of CD8+ T lymphocytes showed detectable autoantibodies (Figs. 6 and 7), but without detectable CTL activity (Fig. 10B). These data also suggest that CD8+ T cells may be involved in a role of the direct tumor killing, whereas CD4+ T
lymphocytes may be required for the induction of both autoantibodies and CD8\(^+\) CTL response to the immunization with hEe-p DNA vaccine.

**Cytokine response**

The spleen cells isolated from the immunized mice or controls were restimulated with syngeneic EGFr-positive tumor cells and supernatants harvested were evaluated for the production of IFN-\(\gamma\) and IL-4 in ELISA. As shown in Fig. 11, the increase in the level of IFN-\(\gamma\) and IL-4 was found in the supernatants harvested from the spleen cells of hEe-p-immunized mice when compared with the controls.

**Discussion**

Several observations have been made in the present study concerning the immunization with DNA vaccine encoding xenogeneic EGFr gene, antitumor immunity, and autoimmune response. The present study has, to our knowledge, first demonstrated that plasmid DNA encoding extracellular domain of human EGFr as vaccine can induce both protective and therapeutic antitumor immunity against EGFr-positive lung cancer and mammary cancer models in mice. Furthermore, our findings suggest that autoreactive immune response against the EGFr-positive tumor cells may be provoked in a cross-reaction with the plasmid DNA encoding xenogeneic EGFr, and that the antitumor activity following xenogeneic EGFr vaccination may be involved in both T cell effectors and autoantibodies. MHC class I-dependent CD8\(^+\) CTL activity can be found in vitro cytotoxicity assay. The depletion of CD8\(^+\) lymphocytes showed partial abrogation of the antitumor activity in vivo. More importantly, the antitumor activity can be acquired with adoptive transfer of CD8\(^+\) T lymphocytes. These findings suggest that MHC class I-dependent CD8\(^+\) CTL-mediated immune response may be partially responsible for the antitumor activity by the vaccination with xenogeneic vaccine. Furthermore, autoantibodies in sera and on the tumor cells were identified. IgG subclasses were substantially increased in response to xenogeneic EGFr vaccine. The inhibition of the in vitro EGFr-positive tumor cell proliferation was found with the purified Igs. The antitumor activity was also acquired with adoptive transfer of the purified Igs. These findings suggest that humoral immunity may also be responsible for the antitumor activity by the vaccination of xenogeneic EGFr vaccine. In addition, the role of the humoral immunity in the antitumor activity is further supported by the present findings that the lack of both CTL activity and autoantibodies by the depletion of CD4\(^+\) T lymphocytes were found to be associated with the complete abrogation of the antitumor activity, whereas the CTL activity by the depletion of CD8\(^+\) lymphocytes was associated with partial abrogation of the antitumor activity. Taken together, the findings mentioned above suggest that both humoral and cellular immune response, including Ab and CTL response, may be required for the antitumor immune response against EGFr-positive tumors with DNA vaccine encoding xenogeneic EGFr gene, but either humoral or cellular immune response alone may play a partial role in the antitumor activity.

The mechanism by which antitumor immune response against EGFr-positive tumors with DNA vaccine encoding xenogeneic EGFr gene in the present study may be involved in both humoral and cellular immunity was also supported by the characteristics of IgG subclass and cytokine response in the present study. It has been reported that CD4\(^+\) T lymphocytes can steer and amplify immune response through the secretion of cytokines and expression of surface molecules such as costimulatory molecules, and that CD4\(^+\) subset cells have distinct functional characteristics (30, 31, 32). Namely, Th1 cells make IFN-\(\gamma\) and are associated with cellular immunity and with IgG switching to Ig2a dominant, while Th2 cells make IL-4 and are associated with humoral immunity and with IgG switching to IgG1 dominant (31, 32). The findings in IgG subclass and cytokine response in the present study suggest that the immunization with hEe-p may be involved in a mixed Th1-/Th2-type immune response. In addition, based on the findings in MHC class I-dependent CD8\(^+\) CTL activity and autoantibodies against EGFr-positive tumor cells in the present study, we may rule out the possibility that antitumor activity with hEe-p may result from the nonspecifically augmented immune response against the tumor growth in host mice.

We found in the present study that in vivo depletion of CD4\(^+\) T lymphocytes could completely abrogate the antitumor activity with the immunization of hEe-p, whereas the depletion of CD8\(^+\) lymphocytes showed partial abrogation of the antitumor activity in vivo. At the same time, mice depleted of CD4\(^+\) T lymphocytes did not develop detectable CTL activity and autoantibodies, whereas mice depleted of CD8\(^+\) T lymphocytes still showed detectable autoantibodies, but without detectable CTL activity. The findings in the present study also showed that CTL and autoantibodies had the antitumor activity by in vitro assay and by the adoptive transfer of CD8\(^+\) lymphocytes or the purified Igs. Thus, these findings may suggest that the complete abrogation of the antitumor activity by the depletion of CD4\(^+\) lymphocytes may result from the failure in the induction of both CTL and autoantibodies, whereas the partial abrogation of the antitumor activity by the depletion of CD8\(^+\) lymphocytes may result from the loss of CD8\(^+\) CTL, but the autoantibody against EGFr is still present and plays a role in its antitumor activity. These suggestions were further supported by the important roles of CD4\(^+\) T lymphocytes in the antitumor immunity (33–39). It has been reported that CD4\(^+\) T cells are required for the generation and maintenance of cytolytic CD8\(^+\) T cells (33, 34) and are generally believed to be essential for the generation of both a cellular and a humoral antitumor immune response (35–39). It has been reported that the MUC1-specific CD4\(^+\) T cell-dependent mechanism of B16.MUC1 tumor protection could be adoptively transferred to wild-type mice or MUC1 transgenic mice, although CD4\(^+\) T lymphocytes-mediated tumor killing activity was not detected in conventional in vitro assays of
cell-mediated cytotoxicity (40). For the Ab-dependent immunity, CD4+ T lymphocytes can be required at the immunization phase as well as at the effector phase (41, 42). Furthermore, CD4+ T lymphocytes have been reported to be required for the induction of antitumor immunity by the vaccination with a recombinant vaccinia virus encoding self tyrosinase-related protein 1 in a mouse melanoma model (42–44). In addition, it has been reported that CD4+ T lymphocytes play a prominent role in classic mouse models of autoimmunity, such as experimental allergic encephalitis, systemic lupus erythematosus, and autoimmune gastritis (45–47). Thus, these findings may help explain the requirement for CD4+ T lymphocytes in the induction of both CTL and autoantibody response against EGFr-positive tumor cells in a cross-reaction in the present study.

Although the therapeutic protocol in the present study is effective in the inhibition of established tumor growth and prolongation of the survival of tumor-bearing mice, it is less successful in treating mice than the prophylactic protocol has shown. Most of the studies also indicate that tumor vaccine is very effective in preventing subsequent tumor challenge, but it is only partially effective against preexisting tumors in mouse models (48). The exact mechanism of the discordant antitumor immune responses generated in tumor-free vs tumor-bearing vaccine recipients has yet to be determined (48), but it has been explained by such diverse factors as the generation of tumor-induced suppressor T cells (49), alterations in T cell signal transduction in the tumor-bearing host (50, 51), tumor induction of T cell apoptosis or anergy (48, 52), the development of peripheral tolerance to tumor Ags (53), or of immunological ignorance (54).

No marked side effects of autoimmunity were found in the immunized mice in the present study. Normally, EGFR number is down-regulated as much as 90% in the presence of EGF (55, 56) through the molecular mechanisms of negative regulation. In contrast, overexpression of EGFR in the tumor cells impairs their down-regulation, apparently because of the limited levels of regulatory molecules that mediate rapid endocytosis and lysosomal targeting (55–58). It has been also reported that EGFR gene expression and function is critical for cancer cell growth, but not for the growth of normal cells (59). In addition, the lung cancer cells and mammary cancer cells used in the present study also showed apparent expression of EGFR and deposition of the autoantibodies, but were without detectable autoantibodies within normal tissues. These findings mentioned above may help explain why there are no marked side effects of autoimmunity found in the present study. Taken together, the findings in the present study may provide a new vaccine strategy for the treatment of EGFr-positive tumors through the induction of the autoimmune response against EGFr in a cross-reaction by the immunization with the plasmid DNA encoding xenogeneic EGFR as vaccine, and may be of importance to the further exploration of the role of the breaking of immune tolerance to self proteins through the cross-reaction between xeno-genic homologous and self molecules in cancer therapy.

References


